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Investigation of Babesia species using molecular methods

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ABSTRACT

Aims: To determine the presence of *Babesia* species in ticks in the Ankara region and neighboring provinces; the aim of this study was to detect and distribute *Babesia* species using molecular methods in ticks collected from nature and hosts.

Methods: *Babesia* was detected at the species level by conventional PCR method by DNA extraction of 191 tick samples collected from Ankara and neighboring provinces, which applied to the Parasitology National Reference Laboratory of the Ministry of Health, General Directorate of Public Health, between 2020-2021.

Results: A total of 191 tick samples were examined in the study, and as a result of the conventional PCR study, 4 *Babesia* agents were found positive, 1 of them was *Babesia* bigemina (*B. bigemina*), 2 were *Babesia* ovis, 1 was *Babesia* spp. were determined.

Conclusion: In tick-borne diseases such as Babesiosis; it should not be forgotten that ticks continue to focus some infections in nature and serve as reservoirs for disease agents in nature, and the necessary strategies for tick control should be followed.

Keywords: *Babesia* spp, tick, parasite

INTRODUCTION

Babesiosis is one of the important diseases that is seen worldwide and is transmitted by ticks and causes medical, veterinary and economic problems. Ticks are ectoparasites that can attach to many living creatures including amphibians, birds, mammals and reptiles with blood-sucking habits and cause the spread of diseases.[1,2] In addition, due to the suitability of climate, surface shape and vegetation, ticks and tick-borne diseases are frequently seen. Ticks and tick-borne diseases are seen at different rates in countries due to reasons such as climate, surface features and suitability of vegetation. Tick-borne diseases are frequently encountered, especially in countries where animal husbandry is high, such as Turkiye.[3]

Tick-borne diseases such as Babesiosis cause a decrease in meat and milk yield, deterioration in wool quality and, most importantly, death, especially in cultured animals. In addition to yield losses, treatment costs also increase the economic dimension of these diseases.[4,5]

Diagnosis of acute Babesiosis is traditionally made by clinical and microscopic methods.[6] Microscopy is insufficient in species identification and low parasitemia. Serological tests such as enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay (IFA), indirect hemagglutination test (IHA), complement fixation (CF) and latex agglutination test (LAT) have been used for a long time in determining subclinical infections and infected animals.[7] Serological tests used in herd screening have disadvantages such as cross-reactivity and false seropositivity due to the presence of antibodies in treated animals. Interest in molecular methods such as polymerase chain reaction (PCR) and reverse line blotting (RLB) has increased since species identification is provided and their sensitivity and reliability are higher.[8,9] Today, molecular methods are widely used in the diagnosis of tick-borne diseases.[10]

In this study, we aimed to diagnose the Babesiosis agent carried by ticks using the PCR method, since microscopic techniques were insufficient to identify the species and the possibility of making a definitive diagnosis was low.

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METHODS

This study was conducted with the decision of Çankırı Karatekin University Science, Maths and Social Sciences Ethics Committee (Date: 09.11.2021, Decision No: 09.11.2021/23). The study was also conducted. In addition, permission was obtained from the head of the Microbiology Reference Laboratories and Biological Products Department for the study. This article based on the master's thesis of Sinem Tunçer (814206/2023).

Sample Collection

Of the 191 ticks collected, 152 were collected from various animals (Sheep, Goat, Marten, Dog, Cat, Rodent, Crocidura), 32 from humans and 5 from nature. Within the scope of the study, ticks were collected considering their seasonal activities. Ticks were collected from suitable habitats in Ankara and its districts (Center, Bala, Beypazarı, Çubuk, Kahramankazan) and Kastamonu, Tokat, Yozgat, Eskişehir, Bartın, Sakarya and Çankırı between March 2020 and October 2021. Those taken from the field were collected by the tick flagging method and from farm animals and dogs. Ticks were identified at the genus and species level microscopically according to their morphological characters.[11] Samples for PCR were stored at -20°C until the study before the experiment.

For PCR, homogenization of ticks is required. In this study, magnetic bead-based extraction method was used and samples were extracted one by one. This method is simple, fast and convenient, effective in removing inhibitors, was preferred in order to produce pure high molecular weight genomic DNA and to obtain more purified products.

DNA extraction from homogenized ticks was performed according to the manufacturer's instructions (Qıagen, Hilden, Germany). Samples were homogenized by adding 500 µl PBS to 1.5 ml Eppendorf tubes, placing them in a pestle-disintegrated state, and vortexing. For tissue lysis, 200 µl ATL buffer and 20 µl proteinase K were added and left at +50°C overnight. For cell lysis, 200 µl AL buffer was added to the lysed sample and slowly withdrawn. Cells were lysed by waiting at 56°C for 10 minutes. 250 µl binding solution and 50 µml magnetic beads were added and incubated at room temperature for 5 minutes. The tubes were placed on a magnetic rack and the liquid in the tube was discarded without touching the magnetic beads. For washing, 600 µl of AW1 buffer was added and mixed on the mixer for 3 minutes, centrifuged and placed on a magnetic rack, and the liquid in the tube was discarded without touching the beads. 700 µl of AW2 buffer was added and mixed on the mixer for 3 minutes, centrifuged and placed on a magnetic rack after centrifugation, and the liquid in the tube was discarded without touching the beads. A second wash was performed with 500 µl of AW2 buffer. After centrifugation, it was placed on a magnetic rack and the liquid in it was discarded again. To get rid of the ethanol in the tube, 700 ml of distilled water was added to the samples in the magnetic rack and immediately withdrawn. 200 µl of AE buffer was added and incubated on the mixer at room temperature. The centrifuged samples were placed on the magnetic rack and the liquid remaining on the side was transferred to clean tubes as DNA extract. DNA extracts were kept at -20 °C until use. Positive control samples were obtained from the Turkiye Republic Health Ministry of Health General Directorate, Microbiology Reference Laboratories and Biological Products Department. In addition, distilled water was used as a negative control. DNA size markers, 100 bp ladder, have been loaded on each side.

In order to identify the presence of *Babesia* species, conventional PCR was performed for the separation of the 18S rRNA protozoa-specific gene region before DNA sequence analysis. For PCR, forward and reverse primers recommended for the 18S rRNA gene region (BJ1- GTCTTGTAATTGGAATGATGG and BN2-TAGTTTATGGTTAGGACTACG primers) were used.[12] Primers were commercially produced (Sentabiolab-Ankara).

After amplification, 1.5% agarose gel (Biomax-EEC European Economic Community) was prepared for the evaluation of amplified products. TBE buffer (Sigma Aldrich Merck Darmstadt/Germany) was used as the running buffer during agarose gel electrophoresis. After the agarose was melted in this buffer, it was cooled to 60^0C, nucleic acid stain (Gelred nucleid acid stain) was added to the agarose gel at a ratio of 1:10000 and poured onto the prepared horizontal gel table. An electrophoresis comb was placed on the agarose poured in a thickness of 5 mm and 30 minutes was waited for the gel to solidify completely. At the end of this period, the comb was removed without damaging the gel. 100 bp DNA ladder (Biomatik Delaware/USA) was loaded into the first well of the gel. 5 µl of amplification products were mixed with 1 µl of loading buffer (Sigma Aldrich Merck Darmstadt/Germany) and loaded onto the gel. The tank lid was closed, a power supply providing direct current to the electrodes was connected, and it was checked whether the movement was towards the anode. The amplified DNA samples were subjected to electrophoresis at 90 volts for 50 minutes. They were visualized under UV light with a gel imaging system (Biostep UST-20M-8X Transilluminator-Germany).

Statistical Analysis

Four samples that were found to be positive for *Babesia* spp. were sent for DNA sequence analysis. Nucleotide analysis was performed using ABI 3730XL sanger sequencer and BigDay Terminator v3.1 cycle sequencing kit to obtain DNA sequence. The obtained data were subjected to NCBI (national center for biotechnology information) blast algorithm and database.

Mega software (version 6.0) was used for phylogenetic analysis.[13] Phylogenetic tree was constructed using the neighbor-joining method based on Kimura.[14] The software used a 2-parameter model.[15] Bootstrap resampling was calculated from 1000 pseudo-replicates with random seeds.[16]

RESULTS

Of the 191 tick samples collected, 95 were male, 85 were female and 11 were nymphs. A total of 4 samples were identified as *Babesia* positive. 81 were identified as *Rhipicephalus sanguineus* (*R. sanguineus* (54♂; 27♀), 6 as *R. bursa* (6♀), 4 as *R. turanicus* (1♂; 3♀), 59 as *Hyalomma marginatum (H. marginatum)* (25♂; 29♀), 5 as nymphs of *Hyalomma* spp., 4 as *H. aegyptium* (3♂; 1♀), 4 as *Haemaphysalis parva* (1♂; 3♀), 8 as *Hae. erinacei* (7♂; 1♀), 1 as *Dermacentor marginatus* (1♂)

Table 1. Samples found positive by molecular analysis (PCR) (n=4)

County	Collected from	Ticks	İnfection Rates					PCR (+)		
			Larva		Nymph	Adult		*Babesia bigemina*	*Babesia ovis*	*Babesia* spp.
			F	M		F	M			
Ankara/Çubuk	Human	*Hyalomama aegyptium*	-	-	-	-	1	+(118)		
Ankara	Human	*Rhipicephalus sanguineus*	-	-	-	-	1		+ (62)	
Eskişehir	Goat	*Rhipicephalus sanguineus*	-	-	-	-	1		+(116)	
Tokat	Crocidura	*Ixodes spp.*	-	-	1	-	-			+(130)

Note: F: Female M: Male. A total of 119 ticks were examined

and 24 as *Ixodes ricinus* (3♂; 15♀), 6 as nymphs of *Ixodes spp.* When the distribution of the collected ticks by province was examined, 37 *R. sanguineus*, 6 *R. bursa*, 2 *R. turanicus*, 33 *H. marginatum*, 2 *H. aegyptium*, 3 *Hae. parva,* 1 *D. marginatus* were found in Ankara. While 14 *I. ricinus* species were found in Sakarya, 8 *Hae. erinacei* and 2 *R. turanicus* were detected in Tokat.

The 18S rRNA gene sequence results obtained with primers determined that samples 62, 116, 118 and 130 were positive for *Babesia* species. One of the positive samples (sample 118) was *Babesia bigemina* (*B. bigemina)*, two (samples 62, 116) were *B. ovis*, and one (sample 130) was *Babesia* spp. One *B. bigemina* was found in a male *H. aegyptium* tick taken from a human in Çubuk district of Ankara, one of the two *B. ovis* was found in a male *R. sanguineus* tick taken from a human in Ankara province, the other was found in a male *R. sanguineus* tick taken from a goat in Eskişehir province, 1 *Babesia* spp, and *Ixodes* spp. was found in a nymph taken from crocidura in Tokat province. Table 1 shows samples that were positive by conventional PCR.

A phylogenetic tree was drawn by comparing the obtained results with the data recorded in NCBI. The phylogenetic tree includes data on the strain, *Babesia* species, the country from which it was obtained, and the species of organism. The sample identified as *Babesi*a spp. (130) is in the same clade as *B. rossi* and *Babesia* spp. The two samples identified as *B. ovis* (62, 116) are in the same clade as the other *B. ovis* cases. *B. bigemina* (118) is in the same clade as the other *B. bigemina*'s.

DISCUSSION

Babesiosis, caused by parasites of the genus *Babesia*, is a worldwide tick-borne zoonotic disease. Ixodes ticks are the primary vectors for the transmission of *Babesia* to vertebrates, including humans. *Babesia* parasites are found in various vertebrate reservoirs, while humans are accidental and terminal hosts. Many different species of *Babesia* parasites have been found in animals, but only a few have been found in humans. *B. microti, B. divergens*, and *B. bovis* are the most common causes of human babesiosis.[17]

In the northeastern and upper Midwestern United States, the main species seen in humans is *B. microti*, with the primary reservoir being the white-footed mouse. *B. microti* is endemic in the United States and sporadic in the rest of the world. In Europe, the bovine parasites *B. bovis* and *B. divergens* have been isolated from human patients, and *B. divergens*

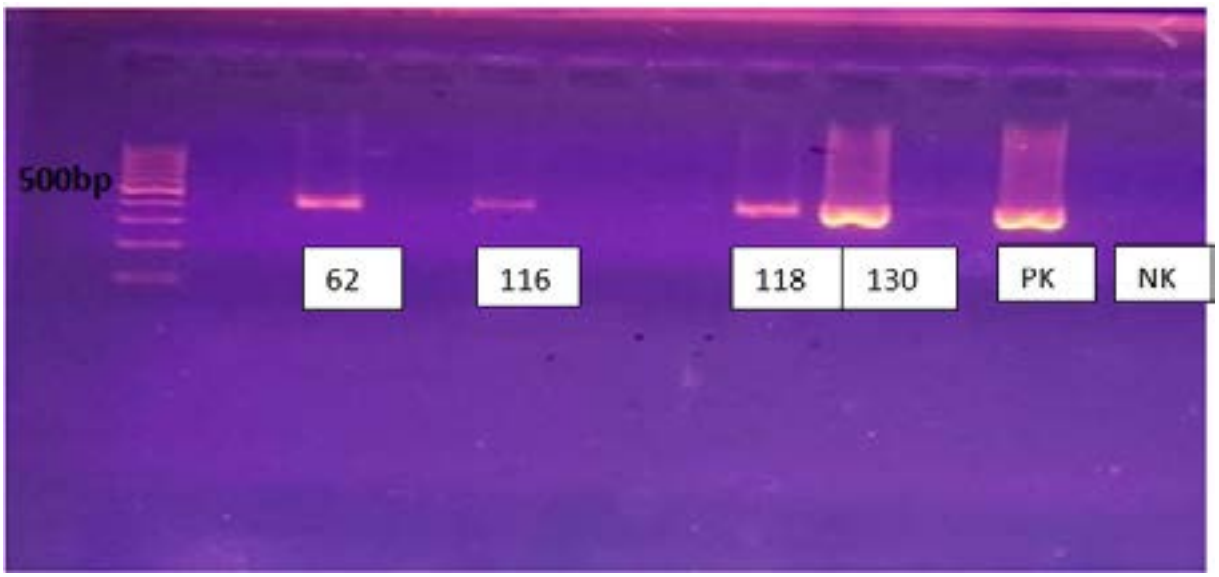

Figure 1. Gel electrophoresis image including samples 62, 116, 118 and 130 after amplification and positive and negative controls. M: marker, PK: positive control, NK: negative control, PO: positive samples (Original)

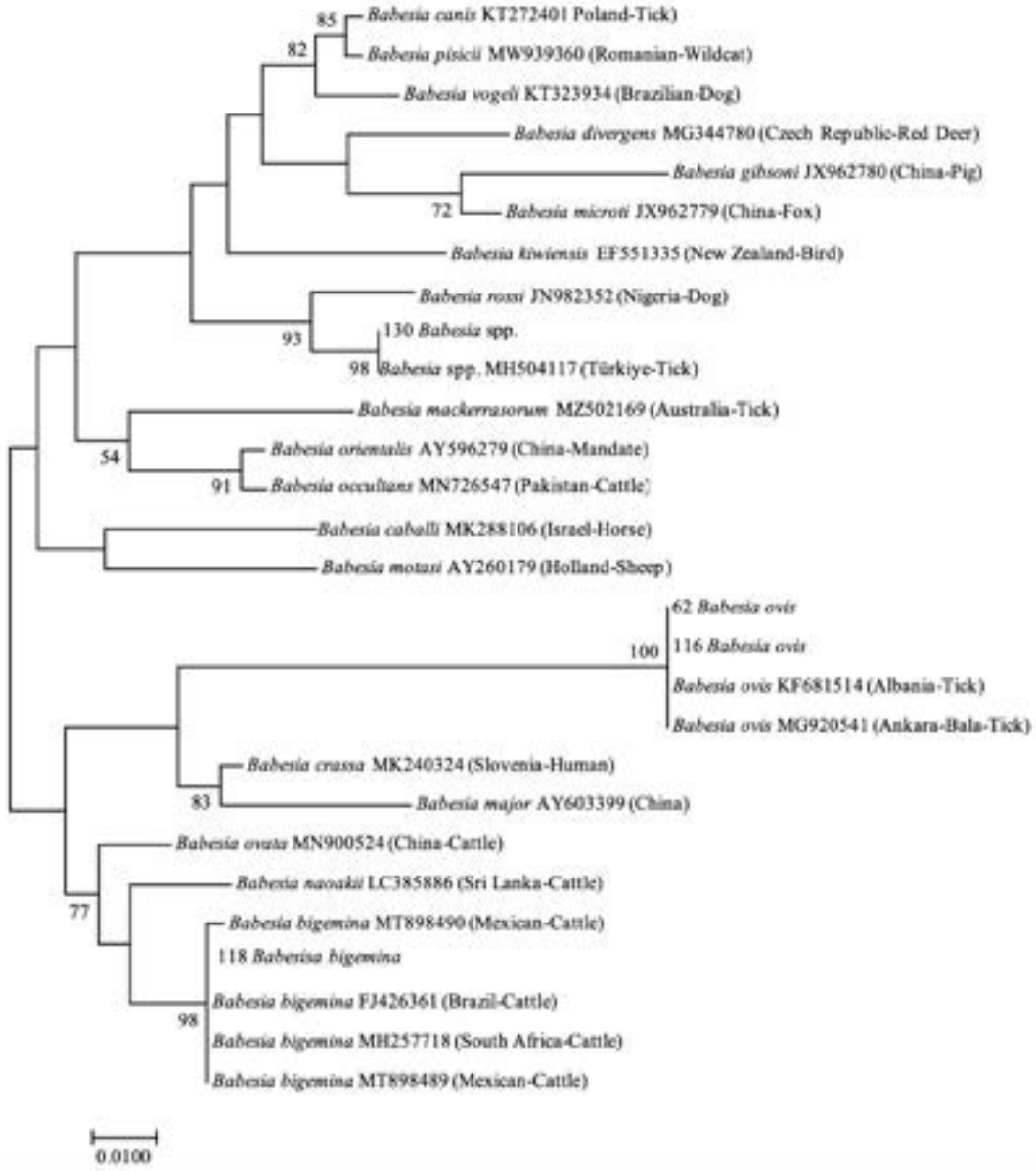

Figure 2. Molecular phylogenetic analysis of Babesia spp. drawn with the maximum likelihood method

infection has generally been detected in individuals who have undergone splenectomy.[18]

The disease occurs especially in the summer months when vector ticks become active. High fever, respiratory distress, pulmonary edema, disseminated intravascular coagulation, renal failure, hemoglobinuria, coma, and splenic rupture are observed in babesiosis. The disease may present itself as a long-term course despite antibiotic treatment. It may progress severely and result in death in patients with a history of travel to endemic areas, in patients who have been exposed to ticks, in older ages, and in patients who have undergone splenectomy. Human babesiosis is most commonly caused by tick bite, but it may also occur through blood transfusion, transplacental, or perinatal transmission.[18,19]

In studies conducted on ticks that infect humans in the Central Anatolian region of Turkiye, *B. bovis, B. ocultans, B. microti*, and *B. ovis* were found.[17,20-22] In our study, *B. microti*, *B. bovis,* and *B. divergens*, which were reported as zoonotic species, could not be determined. *Babesia* parasites have been defined as blood parasites that cause significant economic losses for farm animals, and many studies have reported the presence of *Babesia* species in domestic animals using microscopic and molecular methods. In studies conducted on farm animals in the Central Anatolia, Black Sea, Southeastern Anatolia and Marmara Regions between 2005 and 2020, *B. ovis, B. bigemina* and *Babesia* spp. positivity was detected using PCR and Reverse Line Blotting (RLB) methods.[22-27]

In a study conducted in Izmir, Konya and Gaziantep in 2021 with 152 racehorses, positivity was detected using standard and nested PCR tests. *B. ovis* was found with the sequencing study. This result is the first detection of *B. ovis* DNA in racehorses in Turkiye to date. It should be noted that *B. ovis*, a parasite generally thought to occur in sheep, should also be taken into account in future epidemiological studies on horses.[28]

In studies conducted in Turkiye, ticks are known as the main vectors for *Babesia* species and play an important role in their transmission between animals. In studies conducted in various parts of Turkiye, *B. crassa, B. rossi, B. occultans* and *B. bigemina* were detected in ticks belonging to the genera *Hyalomma* spp., *Haemaphysalis* spp., *Rhipicephalus* spp., *Dermacentor* spp. and *Ixodes* spp using PCR and RLB techniques. *Babesia* spp. and *B. caballi* were found for the first time in horses and *B. vulpes* in foxes. Three of the *Babesia* spp found in our country, suspected to be a new species, were named *Babesia* sp. Rabbit 1, *Babesia* sp. Rabbit 2 and *Babesia* sp. novel (*B. sp ucbas*).[29-33]

Onyiche et al.[34] (2021), found that 3069 *Babesia* species were detected positive in 137.364 ticks in 104 eligible studies from 1985 to 2020. The study shows that global estimates are 2.10%. In total, 19 different *Babesia* species of both human and veterinary importance were detected in 23 tick species. Of the molecular techniques, nested polymerase chain reaction (PCR) was the study with the highest rate at 2.80%. Similarly, in our study, the positivity rate was found to be 2.1% with conventional PCR.[34]

Microscopic examination is widely used in laboratory diagnosis of pathogens in blood smears. However, there are serious problems such as similar morphologies of different parasites, carrier animals with low parasite counts, inability to identify parasites in acute conditions at the beginning of the disease, low sensitivity, need for an expert, and inability to identify species. There are also situations such as cross-reactivity and inability to distinguish active from past infection in serological tests used to detect parasite-specific antibodies. However, antigen-based serological tests can overcome certain limitations. However, there may be difficulties in identifying antigens.[35]

With the advancement of molecular biology, PCR-based diagnostics offer researchers the opportunity to detect and identify a large number of parasites in clinical samples and their natural hosts down to the subspecies or strain level. This innovative method has proven to be the most sensitive and specific method for detecting agents and new strains, eliminating the disadvantages of both microscopy and serology, and is a basic tool for evaluating therapeutic efficacy.[35] Considering all these, conventional PCR was preferred among molecular methods in our study.

CONCLUSION

Four *Babesia* agents were found to be positive (4/191) 2.1%. 1 of these was reported *as B. bigemina*, 2 as *B. ovis* and 1 as *Babesia* spp. Today, the majority of disease agents are of animal origin. The majority of these are zoonotic agents. The limitations of this study may be the inability to collect more ticks. The use of 191 ticks in this study may be due to regional or seasonal collection limitations. Although *Ixodes* ticks are known as summer/pasture ticks, their incidence is high except for a few months of the year. The prevalence of Babesiosis in ticks was found to be lower than in mammals. As in this study, it is thought that keeping regional Babesiosis agent screenings in ticks as up-to-date as possible and following them will provide significant advantages in the control and fight against the disease and agent. It should not be forgotten that ticks act as reservoirs in nature for tick-borne diseases such as Babesiosis and tick control strategies should be followed.

ETHICAL DECLARATIONS

Ethical Approval

This study was conducted with the decision of Çankırı Karatekin University Science, Maths and Social Sciences Ethics Committee (Date: 09.11.2021, Decision No: 09.11.2021/23). The study was also conducted. In addition, permission was obtained from the head of the Microbiology Reference Laboratories and Biological Products Department for the study.

Informed Consent

Informed consent is not required in this bacterial strain study.

Referee Evaluation Process

Externally peer-reviewed.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Financial Disclosure

The authors declared that this study has received no financial support.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

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